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<p>(21) International Application Number: PCT/EP87/00588</p> <p>(22) International Filing Date: 8 October 1987 (08.10.87)</p> <p>(31) Priority Application Numbers: 8624400 8626324</p> <p>(32) Priority Dates: 10 October 1986 (10.10.86) 4 November 1986 (04.11.86)</p> <p>(33) Priority Country: GB</p> <p>(71) Applicant (for all designated States except US): GRUPPO LEPETIT S.P.A. [IT/IT]; Via Murat, 23, I-20159 Milano (IT).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): SELVA, Enrico [IT/IT]; Via Di Vittorio, 15, I-27027 Gropello Cairoli (IT). BERETTA, Grazia [IT/IT]; Via Belgirate, 12, I-20125 Milano (IT). BORGHI, Angelo [IT/IT]; Via Pierluigi da Palestrina, 36, I-20124 Milano (IT). DENARO, Maurizio [IT/IT]; Viale Bligny, 41, I-20136 Milano (IT).</p>		<p>(74) Agent: MACCHETTA, Francesco; Gruppo Lepetit S.P.A., Patent &amp; Trademark Department, Via R. Lepetit, 34, I-21040 Gerenzano (IT).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: GLYCOPEPTIDE ANTIBIOTICS</p> <div data-bbox="389 1218 1347 1701"> </div> <p>(57) Abstract</p> <p>Antibiotic A 40926 complex or a factor thereof are microbially deacylated to produce novel de-acyl A 40926 antibiotics of formula (I) wherein A represents a 2-amino-2-deoxy-beta-D-glucopyranosiduronic acid group and B represents hydrogen, alpha-D-mannopyranosyl or 6-acetyl-alpha-D-mannopyranosyl and the addition salts thereof. The de-acyl A 40926 antibiotics and their addition salts are especially active against gram-positive bacteria.</p>		

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## Glycopeptide antibiotics.

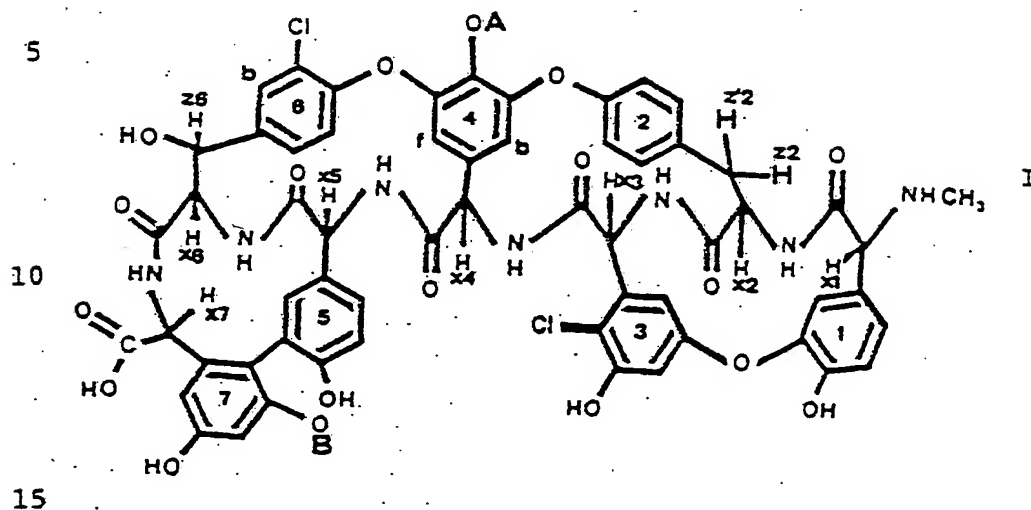
10 Antibiotic A 40926 is a glycopeptidic antibiotic which has been isolated from a culture of Actinomadura, named Actinomadura sp. ATCC 39727. It is a complex whose factors have been named factor A, factor B, factor B<sub>0</sub>, factor PA and factor PB. It was described in EP-A-  
15 177882.

Antibiotic A 40926 can be transformed into the corresponding N-acylaminoglucuronyl aglycon derivatives by acid hydrolysis under controlled conditions as  
20 described in EP 86117452.

Antibiotic A 40926 complex, the factors thereof, the corresponding N-acylaminoglucuronyl aglycon complex and factors thereof, are active mainly against gram  
25 positive bacteria and Neisseriae.

The present invention is directed to new de-acylamino derivatives of the above named compounds, which share the common feature of having an  
30 N-acylaminoglucuronyl group linked to a peptidic moiety through an O-glycosidic bond. They are named de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon and can be represented by the following formula I (the numbering is  
35 analogous to that suggested by Williams J. et al. in J.

Am. Chem. Soc., 106, 4895-4908 (1984) for other glycopeptidic antibiotics):



wherein:

A represents a 2-amino-2-deoxy-beta-D-glucopyranosiduronic acid group and

B represents hydrogen, alpha-D-mannopyranosyl or 6-acetyl-alpha-D-mannopyranosyl,

and the addition salts thereof.

These de-acylated derivatives will be collectively referred to as "de-acyl A 40926 antibiotics" and generically each of them will be referred to as a "de-acyl A 40926 antibiotic".

The above named starting materials, i.e. antibiotic A 40926 complex and factors thereof, the corresponding

N-acylaminoglucuronyl aglycon complex and factors thereof, can be represented by the above formula I wherein A represents a 2-deoxy-2-(C<sub>11</sub>-C<sub>12</sub>)acylamino-beta-D-glucuronyl group and B represents hydrogen, an  
5 alpha-D-mannosyl or 6-acetyl-alpha-D-mannosyl group, or an addition salt thereof.

More particularly, antibiotic A 40926 factor A is the compound of the above formula wherein A represents  
10 2-deoxy-2-undecanoylamino-beta-D-glucopyranosiduronyl and B represents mannosyl, antibiotic A 40926 factor B<sub>0</sub> is the compound of the above formula wherein A represents 2-deoxy-2-isododecanoylamino-beta-D-glucuronyl and B represents alpha-D-mannosyl, antibiotic A 40926 factor  
15 B<sub>1</sub> is the compound of the above formula wherein A represents 2-deoxy-2-dedecanoylamino-beta-D-glucuronyl and B represents alpha-D-mannosyl.

20 Antibiotic A 40926 factors of the "P" series, such as factor PA and factor PB<sub>0</sub>, differ from the corresponding factors (factor A and B<sub>0</sub> respectively), in that the mannose unit is replaced by a 6-acetyl-mannose unit.

25 Antibiotic A 40926 N-acylaminoglucuronyl aglycons are represented by the above formula wherein A is as defined above and B represents hydrogen. Their acyl chain on the aminoglycuronyl group corresponds to those of the single factors of antibiotic A 40926.

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On the basis of the data available and by reference to known substances, one may attribute to de-acyl  
35 antibiotic A 40926 the above formula wherein the A

represents 2-amino-2-deoxy-beta-D-glucuronyl and B represents alpha-D-mannosyl, to de-acyl antibiotic A 40926 P the above formula wherein A represents 2-amino-2-deoxy-beta-D-glucuronyl and B represents 6-acetyl-alpha-D-mannosyl and to antibiotic A 40926 aminoglucuronyl aglycon the above formula wherein A represents 2-amino-2-deoxy-beta-D-glucuronyl and B represents hydrogen.

10 Antibiotic A 40926 factors PA and PB, at least under certain fermentation conditions, are the main antibiotic products of the A 40926 producing microorganism.

15 Antibiotic A 40926 factors A and B are mainly transformation products of antibiotic A 40926 factor PA and factor PB, respectively, and are often already present in the fermentation broth.

20 It has been found that antibiotic A 40926 factor PA can be transformed into antibiotic A 40926 factor A and antibiotic A 40926 factor PB can be transformed into antibiotic A 40926 factor B under basic conditions which lead to the removal of the acetyl group of the mannose unit without displacing the acyl group on the 25 aminoglucuronyl unit.

30 As a consequence, when the fermentation broth, or an antibiotic A 40926 containing extract or concentrate thereof, is allowed to stand for a certain time under basic conditions (e.g. aqueous solution of a nucleophilic base, at a pH >9 overnight,) an antibiotic A 40926 complex will be obtained which is enriched in antibiotic A 40926 factor A and factor B (see EP-A-177882).

The same type of basic transformation can be applied to the conversion of de-acyl antibiotic A 40926 P to de-acyl antibiotic A 40926.

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De-acyl antibiotic A 40926 has the following physico-chemical characteristics:

- 10 A) ultraviolet absorption spectrum, which is shown in Figure 1 of the accompanying drawings, and exhibits the following absorption maxima:

	$\lambda$ max (nm)
15 a) 0.1 M HCl	282
b) phosphate buffer pH 6.0	281
c) phosphate buffer pH 7.4	282, 300 (shoulder)
d) 0.1 M KOH	300

- 20 B) infrared absorption spectrum which is shown in Figure 2 of the accompanying drawings and exhibits the following absorption maxima in nujol mull ( $\nu$ ,  $\text{cm}^{-1}$ ):
- 25 3700-3100; 3000-2800 (nujol); 1650; 1590; 1505; 1460 (nujol); 1375 (nujol); 1300; 1230, 1210, 1150, 1060, 1030, 970, 810, 720 (nujol)

- C)  $^1\text{H}$ -NMR spectrum which is shown in Figure 3 of the accompanying drawings and exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO  $\text{d}_6$  (hexadeuterodimethylsulfoxide) [ $\delta$ , ppm; m; (attributions)]  
2.30, s (N-CH<sub>3</sub>); 2.49, s (DMSO  $\text{d}_5$ ); 2.7-3.8, m (sugar CH's); 2.79 m (Z2); 4.08 m (X6); 4.33 s (X1); 4.37 d (X5); 4.37 d (X7); 4.86 m (X2); 5.08 s (4f); 5.08 s (Z6); 5.27 s (anomeric proton of mannose); 5.35 d (anomeric proton of aminoglucuronic acid); 5.61 d (X4); 5.86 s (4b); 6.05, d (X3); 7.73 s (6b); 6.45-8.49 (aromatic protons and peptidic NH's)  
s = singlet; d = doublet; m = multiplet
- D) Retention time ( $R_t$ ) of 0.34 relative to Vancomycin (Eli Lilly)  
Column: Silanized silica gel Ultrasphere ODS (5  $\mu\text{m}$ )  
4.6 mm x 25 cm Altex (Beckman)  
Isocratic elution with 18 mM sodium phosphate buffer/ $\text{CH}_3\text{CN}$  92/8 (v/v)  
Flow rate: 1.8 ml/min  
Detection: UV 254 nm  
Internal standard: Vancomycin (Eli Lilly)  $R_t$  8.4 min
- E) Molecular weight of 1548 as determined by FAB-MS spectroscopy.
- By comparison with the physico-chemical data of the starting materials with reference in particular to the NMR spectrum, one may note that the peaks corresponding to aliphatic protons in the range 0.8-2.0 ppm are no longer present in the new molecule.



Also in the case of de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon, the main difference between the NMR spectra of these compounds and the corresponding "acylated" ones is the absence of signals of aliphatic protons in the range 0.8-2.0 ppm.

More particularly, the  $^1\text{H}$ -NMR spectrum of deacyl antibiotic A 40926 P have the following groups of signals (ppm) at 270 MHz, recorded in DMSO  $d_6$   $\delta$  ppm, m, (attribution)<sub>7</sub>:

2.0, s ( $\text{CH}_3\text{CO}$ ); 2.3, s ( $\text{NCH}_3$ ); 2.5, s (DMSO  $d_5$ ); 2.7-3.8, m (sugar CH's); 2.8, m (Z2); 4.1, m ( $\text{X}_6$ ); 4.1, m ( $\text{CH}_2\text{O}$ , sugar); 4.4 s ( $\text{X}_1$ ); 4.4 d ( $\text{X}_5$ ); 4.4 d ( $\text{X}_7$ ); 4.9 m ( $\text{X}_2$ ); 5.1, s (4f); 5.1, s, (Z6); 5.3, s (anomeric proton mannose); 5.4, d (anomeric proton aminoglucuronic acid); 5.6, d ( $\text{X}_4$ ); 5.8, s (4b); 6.1 d ( $\text{X}_3$ ); 7.7, s (6b); 6.5-8.6 (aromatic and peptidic NH's).

The  $^1\text{H}$ -NMR spectrum of antibiotic A 40926 aminoglucuronyl aglycon have the following group of signals (ppm) at 270 MHz, recorded in DMSO  $d_6$   $\delta$  ppm, m, (attribution)<sub>7</sub>:

2.3, s ( $\text{NCH}_3$ ); 2.5, s (DMSO  $d_5$ ); 2.7-3.8 m (sugar CH's); 2.8, m (Z2); 4.1, m ( $\text{X}_6$ ); 4.4, s ( $\text{X}_1$ ); 4.4, d ( $\text{X}_5$ ); 4.4 d ( $\text{X}_7$ ); 4.9, m, ( $\text{X}_2$ ); 5.1, s (4f); 5.1, s (Z6); 5.4 d (anomeric proton aminoglucuronic acid); 5.5 d ( $\text{X}_4$ ); 5.7, s (4b); 6.1, d ( $\text{X}_3$ ); 7.7, s (6b); 6.2-8.5 (aromatic and peptidic NH's).

The antibacterial activity of the compounds of the invention can be demonstrated in vitro by means of standard dilution tests on different microorganism cultures..

Culture media and growth conditions for MIC (minimal inhibitory concentration) determinations were as follows: Isosensitest broth (Oxoid), 24 h, for staphylococci, Strep. faecalis and Gram-negative bacteria ( Escherichia coli, Klebsiella pneumoniae); 5 Todd-Hewitt broth (Difco), 24 h for other streptococcal species; GC base broth (Difco) + 1% Isovitalex (BBL), 48 h, CO<sub>2</sub>-enriched atmosphere for Neisseria gonorrhoeae; Brain Heart broth (Difco) + 1% Supplement C (Difco), 48 10 h for Haemophilus influenzae; AC broth (Difco), 24 h, anaerobic atmosphere for Clostridium perfringens; PPLO broth with supplements as in R.T. Evans and D. Taylor-Robinson (J. Antimicrob. Chemother. 4, 57), 24 h for U. urealyticum. Incubation was at 37°C. Inocula were 15 as follows: about 10<sup>4</sup> color-changing units/ml for U. urealyticum; about 10<sup>4</sup>-10<sup>5</sup> colony-forming units/ml for other broth dilution MICs.

The minimal inhibitory concentrations (MIC, microg/ml) for some microorganisms are reported 20 below in Table I.

TABLE I

Strain	M.I.C. (microg/ml) De-acyl Antibiotic A 40926
<u>Staph. aureus</u> L165	1
<u>Staph. aureus</u> (10 <sup>6</sup> cfu/ml)	2
<u>Staph. aureus</u> (30% bovine serum)	2
<u>Staph. epidermidis</u> L147 ATCC 12228 (coagulase negative)	2
<u>Staph. haemolyticus</u> L602 (clinical isolate)	32
<u>Strep. pyogenes</u> L49 C203	0.25
<u>Strep. pneumoniae</u> L44 UC41	0.25
<u>Strep. faecalis</u> L149 ATCC 7080	2
<u>Strep. mitis</u> L796 (clinical isolate)	0.5
<u>Clostridium perfringens</u> L290 ISS 30543	0.13
<u>Neisseria gonorrhoeae</u> L997 ISM68/126	64
<u>Haemophilus influenzae</u> L 970 type b ATCC 19418	128
<u>Escherichia coli</u> L47 SKF 12140	>128
<u>Proteus vulgaris</u> L79 X19H ATCC881	>128
<u>Pseudomonas aeruginosa</u> L4 ATCC10145	>128
<u>Ureaplasma urealyticum</u> L1479 (clinical isolate)	>128
<u>Klebsiella pneumoniae</u> L142	>128

Antibiotic A 40926 aminoglucuronyl aglycon and de-acyl antibiotic A 40926 P show substantially the same level of antimicrobial activity as that reported above for de-acyl antibiotic A 40926.

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The antimicrobial activity of the compounds of the invention is confirmed also in experimental septicemia in the mice.

Control and treatment groups may include ten CD-1 mice (Charles River) weighing 18-22 g. They are infected intraperitoneally with 0.5 ml of bacterial suspension prepared by diluting an overnight culture of S. pyogenes C 203 (L 49) with sterile peptonized saline. Inocula are adjusted so that untreated animals die of septicemia within 48 h. The compounds to be tested are administered subcutaneously immediately after infection. On the 7th day, the ED<sub>50</sub> in mg/kg is calculated by the method of Spearman and Kärber (D.J. Finney "Statistical Methods in Biological Assay", Griffin, page 524, 1952) from the percentage of surviving animals at each dose.

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For example, under these conditions the ED<sub>50</sub> of de-acyl antibiotic A 40926 is 2.33 mg/kg, s.c.

The de-acyl A 40926 antibiotics possess acid and basic functions and can form salts with organic and inorganic counter ions according to conventional procedures.

Representative and suitable acid addition salts of the compounds of the invention include those salts formed by standard reaction with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic, glutamic, camphoric, glutaric, glycolic,

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phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic and the like acids.

Representative examples of these bases are: alkali metal or alkaline-earth metal hydroxide such sodium, potassium, calcium, magnesium, barium hydroxide; ammonia and aliphatic, alicyclic or aromatic organic amines such as methylamine, dimethylamine, trimethylamine, and picoline.

The transformation of the "non-salt" compounds of the invention into the corresponding addition salts, and the reverse, i.e. the transformation of an addition salt of a compound of the invention into the non-salt form, are within the ordinary technical skill and are encompassed by the present invention.

For instance de-acyl antibiotic A 40926, antibiotic A 40926 aminoglucuronyl aglycon or de-acyl antibiotic A 40926 P can be transformed into the corresponding acid or base addition-salt by dissolving the non-salt form in an aqueous solvent and adding a slight molar excess of the selected acid or base. The resulting solution or suspension is then lyophilized to recover the desired salt.

In case the final salt is insoluble in a solvent where the non-salt form is soluble it is recovered by filtration from the organic solution of the non-salt form after addition of the stoichiometric amount or a slight molar excess of the selected acid or base.

The non-salt form can be prepared from a corresponding acid or base salt dissolved in an aqueous solvent which is then neutralized to free the non-salt form.

When following this step, the elimination of an excess of acid or base is necessary, a common desalting procedure may be employed.

For example, column chromatography on silanized  
5 silica gel, non-functionalized polystyrene, acrylic and controlled pore polydextrane resins (such as Sephadex LH 20) or activated carbon may be conveniently used. After eluting the undesired salts with an aqueous solution, the desired product is eluted by means of a  
10 linear gradient or a step-gradient of a mixture of water and a polar or apolar organic solvent, such as acetonitrile/water from 50:50 to about 100% acetonitrile.

15 As it is known in the art, the salt formation either with pharmaceutically acceptable acids (or bases) or non-pharmaceutically acceptable acids (or bases) may be used as a convenient purification technique. After formation and isolation, the salt form of an A 40926  
20 antibiotic can be transformed into the corresponding non-salt form or into a pharmaceutically acceptable salt form.

In some instances, a base addition salt of a  
25 de-acyl A 40926 antibiotic is more soluble in water and hydrophilic solvents.

The de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon  
30 are prepared from antibiotic A 40926 complex or a factor thereof, antibiotic A 40926 factor PA or factor PB or a mixture thereof, and antibiotic A 40926 N-acylaminoglucuronyl aglycon complex or a factor thereof, respectively, by a microbiological transformation with suitable  
35 Actinoplanes strains such as Actinoplanes

teichomyceticus ATCC 31121, Actinoplanes missouriensis ATCC 23342, Actinoplanes missouriensis NRRL 15647 or NRRL 15646, and Actinoplanes NRRL 3884. Actinoplanes teichomyceticus ATCC 31121 is described in U.S. patent 4,239,751, Actinoplanes missouriensis ATCC 23342 is described in U.S. patent 3,952,095, Actinoplanes missouriensis NRRL 15647 and NRRL 15646 are described in U.S. patent 4,587,218, while Actinoplanes NRRL 3884 is described in U.S. patent 3,780,174. All these strains are available from the respective culture collections.

More particularly, the selected starting material, either in pure form or in the form of any crude preparations thereof, including harvested fermentation broth of Actinomadura sp. ATCC 39727 or a producing mutant or variant thereof, is contacted with a culture of an Actinoplanes strain such as Actinoplanes teichomyceticus ATCC 31121, Actinoplanes missouriensis ATCC 23342, Actinoplanes missouriensis NRRL 15646, Actinoplanes missouriensis NRRL 15647 or Actinoplanes NRRL 3884, preferably during fermentation.

An Actinoplanes strain, such as preferably, Actinoplanes teichomyceticus ATCC 31121, Actinoplanes missouriensis ATCC 23342, Actinoplanes missouriensis NRRL 15646, Actinoplanes missouriensis NRRL 15647 or Actinoplanes NRRL 3884, are cultivated under usual submerged aerobic conditions in a medium containing assimilable sources of carbon, nitrogen and inorganic salts. Examples of such media are those reported in the above cited U.S. patents and those generally known in the art.

Generally, the starting material mentioned above can be added to a culture of an Actinoplanes strain such as preferably Actinoplanes teichomyceticus ATCC 31121,

Actinoplanes missouriensis ATCC 23342, Actinoplanes missouriensis NRRL 15646, Actinoplanes missouriensis NRRL 15647 or Actinoplanes NRRL 3884, at a time varying from time zero to the time at which the culture has reached its maximum growth. Addition after 36-72 h of growth is, at least in some instances, preferred.

The reaction temperature is generally between 20°C and 40° and preferably between 24°C and 35°C and most preferably between 25°C and 32°C.

The reaction time, i.e. the time of exposure of the starting material to the microbial culture environment before recovering the final product, may vary between 100 and 300 h, depending on the specific conditions employed. Anyway, since the reaction can be monitored as known in the art, for instance by following the decrease of the starting material and/or the increase of the final product by HPLC, the skilled man is capable of readily determine when the reaction is to be considered as complete and the recovery procedure can be started.

Instead of employing a growing culture of an Actinoplanes strain such as Actinoplanes teichomyceticus ATCC 31121, Actinoplanes missouriensis ATCC 23342, Actinoplanes missouriensis NRRL 15646, Actinoplanes missouriensis NRRL 15647 or Actinoplanes NRRL 3884, one may employ a culture of any mutant or variant thereof which is still capable of de-acylating the above mentioned starting material to give the de-acylated compounds of the invention. Any process according to the present invention which employs any such mutant or variant, is considered to be encompassed by the scope of the present invention. Actually, Actinoplanes missouriensis NRRL 15646 and NRRL 15647 are obtained by chemical mutagenesis of Actinoplanes missouriensis ATCC 31683 which is in turn a mutation product of Actinoplanes missouriensis ATCC 23342. Actinoplanes



5 missouriensis ATCC 31683 is described in U.S. patent 4,322,406 and 4,375,513 with Actinoplanes missouriensis ATCC 31682 and ATCC 32680 and is available from the culture collection as the other mentioned Actinoplanes strains.

A mutant strain of Actinoplanes teichomyceticus ATCC 31121 was deposited on July 21, 1987 with ATCC where it received accession number 53649. This strain was deposited under the provisions of the Budapest  
10 Treaty.

Instead of using single pure cultures of the above deacylating microorganisms, one may use a mixture thereof in any proportion.

15 The compounds of the present invention can be prepared according to the method of the invention also by using the washed mycelium of one of the above identified de-acylating microorganism cultures,  
20 conveniently re-suspended in a physiologically acceptable medium, a cell-free preparation obtained by disrupting the cells, e.g. by sonication and collecting the debris by centrifugation, or a cell-free water soluble extract or concentrate obtained from a disrupted  
25 cell preparation. Reaction time and temperature may require a certain adaptation in this case, but substantially mirror those indicated above for the whole microbial culture, even if the temperature may be increased, at least in some instances, up to 50°-60°C,  
30 and preferably is between 25°C and 50°C.

The recovery of the antibiotic substances from the reaction medium is then conducted according to known per se techniques which include extraction with solvents,  
35 precipitation by adding non-solvents or by changing the pH of the solution, partition chromatography,

reverse-phase partition chromatography, ion-exchange chromatography, affinity chromatography and the like.

5 A preferred procedure includes an affinity chromatography on immobilized D-Alanyl-D-Alanine followed by separation at a different pH.

10 Immobilized D-Alanyl-D-Alanine matrices suitable for the present recovery process are disclosed in European Patent Application Publication No. 122969. The preferred matrix in this recovery process is D-Alanyl-D-Alanine coupled with a controlled pore cross-linked polydextrane which is also described therein.

15

The reaction medium can be subjected to the affinity chromatography directly after filtration or after a preliminary purification procedure. This latter procedure includes making the whole medium basic, preferably between pH 8.5 and 10.5 and then filtering in the presence of a filter aid, if convenient. If the reaction medium is kept for a certain time at basic pH de-acyl antibiotic A 40926 P is transformed into de-acyl antibiotic A 40926 analogously to the transformation, under the same conditions, of the respective starting materials. (This transformation can be monitored by HPLC as usual).

20 The clear filtrate is then adjusted to a pH value between 7 and 8 and then subjected to an affinity chromatography on immobilized D-Alanyl-D-Alanine, either in column or batchwise.

30 While the binding of the substance to the affinity matrix is preferably made at a pH of about 7.0-8.0, its elution is performed at more basic pH values (preferably

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between 9.0 and 10.5) by means of an aqueous base. This aqueous base may be ammonia, a volatile amine, an alkali or alkali metal hydroxide or a basic buffered solution optionally in the presence of a polar organic solvent  
5 such as a polar water-miscible solvent.

Representative examples of polar water-miscible solvents are: water-soluble alcohols, (such as methanol, ethanol, iso-propanol, n-butanol), acetone, acetonitrile, lower alkyl alkanoates (such as ethyl  
10 acetate), tetrahydrofuran, dioxane and dimethylformamide and mixtures thereof; the preferred polar water-miscible solvent being acetonitrile.

After removing the impurities by rinsing the column with aqueous buffer pH 4-9, optionally containing salts, urea and/or water-miscible solvents, the de-acyl A 40926  
15 antibiotic substance is eluted with the above eluting mixture.

This eluate is adjusted to pH 2.5-4.0 with an organic or mineral acid to remove the materials which  
20 are insoluble at this pH.

The precipitate is removed by filtration or centrifugation and the supernatant containing de-acyl A 40926 antibiotic is then conveniently desalted.

25 A convenient desalting procedure includes applying the antibiotic containing aqueous solution to a silanized silica gel column, washing with distilled water and eluting with a mixture of a polar water-miscible solvent as defined above and water.

30 Alternatively, desalting may be carried out by applying the antibiotic containing solution to the above described affinity column, washing with distilled water and eluting with a volatile aqueous base as described above for the elution of the affinity chromatography.

The obtained product, namely de-acyl A 40926 antibiotic, antibiotic A 40926 aminoglucuronyl aglycon or de-acyl antibiotic A 40926 P, is obtained substantially pure by concentrating the eluted fractions  
5 containing it (HPLC analysis) followed by precipitation by addition of a non-solvent or lyophilization.

Examples of non-solvents are water miscible ketones such as acetone or methylethyl ketone, or water-miscible alcohols such as methanol, ethanol, propanol and the  
10 like, as well as their mixtures with water-miscible organic solvents such as petroleum ether, lower alkyl ethers, such as ethyl ether, propyl ether and butyl ether.

De-acyl antibiotic A 40926, de-acyl antibiotic  
15 A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon are active against gram-positive bacteria which are responsible for many widely diffused infections. Because of the increasing resistance of these pathogens to the usual therapeutic treatments, the need for new  
20 antibiotic substances is still great.

In general, for antibacterial treatment de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon as well as  
25 the non-toxic pharmaceutically acceptable salts thereof or mixture thereof, can be administered by different routes such as topically or parenterally. The parenteral administration is, in general, the preferred route of administration.

30 Compositions for injection may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain adjuvants such as suspending, stabilizing and/or dispersing agents.

Alternatively, the active ingredient may be in  
35 powder form for reconstitution at the time of delivery

when a suitable vehicle, such as sterile water, is added thereto.

Depending on the route of administration, these compounds can be formulated into various dosage forms.

5 In some instances, it may be possible to formulate the compounds of the invention in enteric-coated dosage forms for oral administration which may be prepared as known in the art (see for instance "Remington's Pharmaceutical Sciences", fifteenth edition, Mack  
10 Publishing Company, Easton, Pennsylvania, USA, page 1614).

This could be specially the case when the absorption of the antimicrobial substance in the enteric tract is particularly desired while passing unaltered  
15 through the gastric tract.

The amount of active principle to be administered depends on various factors such as the size and condition of the subject to be treated, the route and  
20 frequency of administration, and the causative agent involved.

The antibiotic substances of the present invention, namely de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon  
25 and the physiologically acceptable salts thereof, are generally effective at a daily dosage of between about 0.5 and 50 mg of active ingredient per kilogram of patient body weight, optionally divided into 1 to 4 administrations per day.

30 Particularly desirable compositions are those prepared in dosage units containing from about 100 to about 5,000 mg per unit.

Sustained-action formulations can be prepared based on different mechanisms and methods, as known in the art.

A preferred method for preparing a sustained-action formulation containing de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P or antibiotic A 40926 aminoglucuronyl aglycon, involves the use of a water insoluble form of the antibiotic suspended in an aqueous or oily medium.

Preferably, the pharmaceutical preparations of the invention, are intended for therapy (including prevention, treatment, cure, etc.) in humans, even if primates and mammals in general as well as pet animals can also be treated with the compounds and preparations of the invention.

Preparation of pharmaceutical compositions:

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 500 mg of a physiologically acceptable base addition salt of de-acyl antibiotic A 40926

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 500 mg of a physiologically acceptable base addition salt of antibiotic A 40926 aminoglucuronyl aglycon.

A unit dosage form for intramuscular injection is prepared with 5 ml of sterile suspension USP containing 8% propylene glycol and 250 mg of a physiologically acceptable base addition salt of antibiotic A 40926 aminoglucuronyl aglycon.

A unit dosage form for intramuscular injection is prepared with 1,000 mg of antibiotic A 40926 aminoglucuronyl aglycon in the water-insoluble form suspended in 5 ml of sterile water for injection.

Furthermore, the antibiotic substances of the invention can be useful for suppressing the growth of Clostridium difficile which causes pseudomembranous colitis in the intestine. These antibiotics could be used in the treatment of pseudomembranous colitis by the oral administration of an effective dose of the antibiotics or a pharmaceutically-acceptable salt thereof, prepared in a pharmaceutically-acceptable dosage form. For such use, the antibiotics can be administered in gelatin capsules or in liquid suspension.

Besides their activity as medicaments, de-acyl antibiotic A 40926, de-acyl antibiotic A 40926 P and antibiotic A 40926 aminoglucuronyl aglycon and the pharmaceutically acceptable salts thereof, can be used as animal growth promoters.

The term "animal" in this context, is intended to encompass any non-human warm-blooded animal, in particular those bred ultimately as a source material for human consumption, and pet animals.

For this purpose, a compound of the invention is administered orally in a suitable feed. The exact

concentration employed is that which is required to provide for the active agent in a growth promotant effective amount when normal amounts of feed are consumed.

5       The addition of the active compound of the invention to animal feed is preferably accomplished by preparing an appropriate feed premix containing the active compound in an effective amount and incorporating the premix into the complete ration.

10       Alternatively, an intermediate concentrate or feed supplement containing the active ingredient can be blended into the feed.

15       The way in which such feed premixes and complete rations can be prepared and administered are described in reference books (such as "Applied Animal Nutrition", W.H. Freedman and CO., S. Francisco, USA, 1969 or "Livestock Feeds and Feeding" O and B books, Corvallis, Oregon, USA, 1977) and are incorporated herein by reference.

20       The preparation of antibiotic A 40926 complex and the single factors thereof from Actinomadura sp. ATCC 39727 or a producing mutant or variant thereof is described in EP-A- 177882.

25       Preparation of antibiotic A 40926 N-acylaminoglucuronyl aglycons:

30       Antibiotic A 40926 N-acylaminoglucuronyl aglycon complex AB, N-acylaminoglucuronyl aglycon factor A, N-acylaminoglucuronyl aglycon factor B, antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B<sub>0</sub>, antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B<sub>1</sub> and antibiotic A 40926 aglycon are prepared from  
35       antibiotic A 40926 complex or a single factor or mixture



of said factors in any proportion, i.e. A 40926 factor A, A 40926 factor B, A 40926 factor PA, A 40926 factor PB, A 40926 factor B<sub>0</sub> and A 40926 factor B<sub>1</sub>, by controlled acid hydrolysis.

5        Generally, this hydrolysis is conducted in the presence of a strong acid in a suitable organic solvent. The reaction temperature may vary considerably; preferably it is between 4°C and 100°C and most preferably between 25°C and 80°C.

10       The reaction time varies depending on the specific reaction conditions.

         Generally, the reaction time is between 30 min and 120 h.

15       However, since the reaction course may be monitored by TLC or HPLC, the skilled man is capable of deciding when the hydrolysis of the starting materials is to be considered as completed and the recovery procedure may be started.

20       Representative examples of strong acids are mineral or organic strong acids such as hydrogen halides, e.g. hydrogen chloride, bromide and iodide, phosphoric acids, sulfuric acid, haloacetic acids, e.g. trichloroacetic acid, trifluoroacetic acid, chlorodifluoroacetic acid  
25       and the like.

         Suitable organic solvents are such that:

- 30       a) they may at least partially solubilize the starting materials;  
         b) the products, once obtained, either separate or may be separated from them according to usual techniques, and  
         c) in any case, they do not unfavorably interfere with the reaction course.

Examples of said organic solvents are protic or aprotic solvents such as (C<sub>1</sub>-C<sub>4</sub>)alkyl sulfoxides, e.g. dimethylsulfoxide and diethylsulfoxide, (C<sub>1</sub>-C<sub>4</sub>)alkyl formamides, e.g. dimethylformamide, diethylformamide, 5 dioxane, tetrahydrofuran and similar solvents, which are of course compatible with the selected acid.

10 In general, the hydrolysis is conducted in the presence of a limited amount of water, e.g. from 0.1 to 10% (w/w) of the reaction mixture. This amount of water can obviously be already present either in the starting materials, solvents and/or reagents, or may be added ad hoc, if necessary.

15 A preferred embodiment of this process is represented by the use of a mixture dimethylsulfoxide/concentrated hydrochloric acid at a temperature between 40°C and 80°C. Typically, the ratio of the mixture dimethylsulfoxide/concentrated 20 hydrochloric acid is from 8:2 to 9.5:0.5. Preferred concentrated hydrochloric acid is 37% (w/w) hydrochloric acid.

25 Generally, the reaction product is a mixture of the N-acylaminoglucuronyl aglycons and the aglycon. By controlling the temperature, and in some instances also the concentration and strength of the acid, it is possible to direct the process, at least to a certain extent, to the production of one of the two main 30 products, i.e. antibiotic A 40926 N-acylaminoglucuronyl aglycons or antibiotic A 40926 aglycon. More particularly, by keeping a comparatively low temperature, possibly reducing the strength of the acid mixture and properly controlling the reaction time, the 35 yields in the N-acylaminoglucuronyl aglycons are

increased, while at comparatively higher temperatures and longer times the aglycon alone is obtained.

Also in this case, the reaction course is monitored  
5 by TLC or preferably HPLC and the reaction may be stopped when the optimal production of the desired substance is obtained in order to maximize the yields of the subsequent recovery process.

10 When a product is obtained which is a mixture of antibiotic A 40926 N-acylaminoglucuronyl aglycons and antibiotic A 40926 aglycon it can be separated by chromatography such as liquid/liquid chromatography, flash chromatography, high pressure liquid  
15 chromatography and affinity chromatography.

When affinity chromatography is used, a preferred adsorbent is an immobilized D-Alanyl-D-Alanine as described in EP-A- 122969. Particularly preferred is  
20 agarose-epsilon-aminocaproyl-D-Alanyl-D-Alanine. The elution mixture is a mixture of an aqueous buffer and a saline solution. By adjusting the pH and the salt concentration antibiotic A 40926 N-acylaminoglucuronyl aglycons are separated from antibiotic A 40926 aglycon.

25

A preferred procedure for prevalently preparing antibiotic A 40926 N-acylaminoglucuronyl aglycon complex AB or a factor thereof is a process which comprises  
subjecting antibiotic A 40926 complex or a single factor  
30 thereof, antibiotic A 40926 complex AB, antibiotic A 40926 factor A, antibiotic A 40926 factor B, antibiotic A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor B<sub>1</sub>, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB to controlled acid hydrolysis with a mixture  
35 of a polar aprotic solvent and a strong mineral or

organic acid in the presence of a limited (0.1-10%, w/w) amount of water at a temperature between room temperature and 100°C and preferably between 40°C and 65°C for a time of from 3 h to 120 h.

5        Most preferably the hydrolyzing mixture is a mixture of dimethylsulfoxide and 37% hydrochloric acid from 9:1 to 9.5:0.5, the temperature is 65°C and the reaction time is 5 h.

10        When the starting material for the preparation of the N-acylaminoglucuronyl aglycon is antibiotic A 40926 complex, a final product is obtained which is still a mixture of factors substantially corresponding to those of the original complex, while when a single factor is  
15        used, such as antibiotic A 40926 factor A or factor B, a single N-acylaminoglucuronyl aglycon factor is obtained which is respectively antibiotic A 40926 N-acylaminoglucuronyl aglycon factor A and antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B (which  
20        can in turn be separated into factor B<sub>0</sub> and B<sub>1</sub>).

When an antibiotic A 40926 N-acylaminoglucuronyl aglycon complex AB is obtained, it can be separated into its single factors by known per se techniques such as  
25        liquid/liquid chromatography and preferably preparative HPLC.

A preferred procedure includes reverse-phase liquid chromatography, preferably in stainless steel columns  
30        under moderate pressure (5-50 bar) or at high pressure (100-200 bar). The solid phase may be a silanized silica gel with a hydrocarbon phase at (2-18) carbon atoms (most preferably C 18) or phenyl group and the eluent is a mixture of a polar water-miscible solvent as defined

above and an aqueous buffer at a pH compatible with the resin (preferably pH 4-8).

Most preferred is a linear gradient elution mixture  
5 of a polar water soluble aprotic solvent selected from acetonitrile and an aqueous buffer solution at pH between 4 and 8 and preferably about 6, such as a linear gradient from 5% to 45% of a mixture  
10 acetonitrile/phosphate buffer, pH 6, 70:30 and a mixture acetonitrile/phosphate buffer, pH 6, 10:90.

Antibiotic A 40926 N-acylaminoglucuronyl aglycon complex AB (in the non-addition salt form) has the following characteristics:

15

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

		$\lambda$ max (nm)
20	a) 0.1 N HCl	282
	b) phosphate buffer pH 7.4	282
		310 (shoulder)
	c) 0.1 N KOH	302

25 B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
3700-3100; 3000-2800 (nujol); 1650; 1620-1550;  
1500; 1460 (nujol); 1375 (nujol); 1300; 1250-1180;  
1150; 1060; 1010; 970; 930; 840, 820

30

C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in  $\text{DMSO } d_6$  (hexadeuterodimethylsulfoxide) plus  $\text{CF}_3\text{COOH}$  using TMS as the internal standard (0.00 ppm), ( $\delta$  = ppm):

0.84, d and t /isopropyl CH<sub>3</sub>'s and terminal  
 CH<sub>3</sub>-; 1.14, m /-(CH<sub>2</sub>)<sub>n</sub>-; 1.44, m /-CH<sub>2</sub>-C-CO and  
 isopropyl CH-; 2.00, t /-CH<sub>2</sub>-(CO)-; 2.5 s  
 (DMSO-d<sub>5</sub>); 2.5 s (N-CH<sub>3</sub>); 2.93, m /-CH, (Z2)-; 3.33,  
 5 m /-CH, (Z'2)-; 3.20-3.80, m /sugar CH's-; 5.34, d  
 /anomeric proton of acylaminoglucuronic acid-; 4.10  
 m (X6); 4.33 d, (X5); 4.43 d (X7); 4.9 m (X2); 5.1  
 (4f and Z6); 5.4 s (X1); 5.58 d (X4); 5.7 s (4b);  
 6.06 d (X3); 7.73 s (6b); 6.26-8.42 s and m  
 10 /aromatic CH's and peptidic NH's-; 8.70-10.5, br s  
 /phenolic OH's and NH<sub>2</sub><sup>+</sup>-

br = broad  
 d = doublet  
 15 m = multiplet  
 s = singlet  
 t = triplet

D) Retention times (R<sub>t</sub>) of 1.20 and 1.30 relative to  
 20 Teicoplanin A<sub>2</sub> component 2 (R<sub>t</sub> = 20.3 min) when  
 analyzed by reverse phase HPLC under the following  
 conditions:

column: Ultrasphere ODS (5 μm) Altex (Beckman)  
 25 4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5 μm)

eluent A: CH<sub>3</sub>CN 10% } adjusted at  
 30 (2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 90% } pH 6.0

eluent B: CH<sub>3</sub>CN 70% } adjusted at  
 (2.5 g/l) NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

5

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

10

E) acid functions capable of forming salts

F) amino function capable of forming salts

15 G) no mannose unit linked to the core moiety.

20 Antibiotic A 40926 N-acylaminoglucuronyl aglycon  
factor A (in the non-addition salt form) has the  
following characteristics:

25 A) ultraviolet absorption spectrum which exhibits the  
following absorption maxima:

	$\lambda_{\text{max}}$ (nm)
a) 0.1 N HCl	282
b) phosphate buffer pH 7.4	282
	310 (shoulder)
30 c) 0.1 N KOH	302

B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):

3700-3000; 3000-2800; 1650; 1585; 1505; 1460  
(nujol); 1375 (nujol); 1295; 1230; 1210; 1150;  
5 1070; 1060; 1010; 845; 820; 720 (nujol)

C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in  $\text{DMSO } d_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta = \text{ppm}$ ):

10 0.85 t (terminal  $\text{CH}_3$ ); 1.0  $\div$  1.3 (aliphatic  $\text{CH}_2$ 's);  
1.42 m ((OC-C) $\text{CH}_2$ ); 2.00 t ((CO) $\text{CH}_2$ ); 2.35 s  
( $\text{NCH}_3$ ); 2.49 s ( $\text{DMSO } d_5$ ); 2.82 m (Z2); 2.8  $\div$  3.8  
(sugar protons and Z'2); 4.12 m (X6); 4.56 s (X1);  
15 4.34 d (X5); 4.41 d (X7); 4.96 m (X2); 5.08 - 5.12  
(4f and Z6); 5.40 d (anomeric proton of  
acylaminoglucuronic acid); 5.58 d (X4); 5.74 s  
(4b); 6.05 d (X3); 7.75 s (6b); 6.25-8.40 s, d and  
m (aromatic  $\text{CH}$ 's and peptidic  $\text{NH}$ 's)

20 D) Retention time ( $R_t$ ) of 1.20 relative to Teicoplanin  
 $A_2$  component 2 ( $R_t = 20.3 \text{ min}$ ) when analyzed by  
reverse phase HPLC under the following conditions:

25 column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm

pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )

30 eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0

eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

35



elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

5

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

10

E) Molecular weight of about 1554 as determined by  
FAB-MS

F) acid functions capable of forming salts

15

G) amino function capable of forming salts

H) no mannose unit linked to the core moiety.

20

Antibiotic A 40926 N-acylaminoglucuronyl aglycon  
factor B<sub>0</sub> (in the non-addition salt form) has the  
following characteristics:

25

A) ultraviolet absorption spectrum which exhibits the  
following absorption maxima:

30

	$\lambda$ max (nm)
a) 0.1 N HCl	282
b) phosphate buffer pH 7.4	282
	310 (shoulder)
c) 0.1 N KOH	302

- B) infrared absorption spectrum which exhibits the following absorption maxima ( $\text{cm}^{-1}$ ):  
3700-3100; 3000-2800 (nujol); 1650; 1585; 1505;  
1460 (nujol); 1375 (nujol); 1295; 1230; 1210; 1150;  
1060; 1010; 980; 840; 820; 720 (nujol)
- C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO  $\text{d}_6$  (hexadeuterodimethylsulfoxide) using TMS as the internal standard (0.00 ppm), ( $\delta = \text{ppm}$ ):  
0.84, d (isopropyllic  $\text{CH}_3$ 's); 1.0  $\div$  1.3 (aliphatic  $\text{CH}_2$ 's); 1.3  $\div$  1.6 ((OC-C)- $\text{CH}_2$  and isopropyllic -CH);  
2.00 t ((OC) $\text{CH}_2$ ); 2.32 s ( $\text{NCH}_3$ ); 2.49 s ( $\text{DMSO-d}_5$ );  
2.82 m (22); 2.9  $\div$  3.8 (sugar protons); 4.12 m (X6); 4.44 s (X1); 4.33 d (X5); 4.37 d (X7); 4.95 m (X2); 5.06  $\div$  5.10 (4f and Z6); 5.38 d (anomeric proton of acylaminoglucuronic acid); 5.59 d (X4); 5.72 s (4b); 6.05 d (X3); 7.74 s (6b); 6.27  $\div$  8.5 (aromatic and peptidic NH's)
- D) Retention time ( $R_t$ ) of 1.30 relative to Teicoplanin  $A_2$  component 2 ( $R_t = 20.3$  min) when analyzed by reverse phase HPLC under the following conditions:
- column: Ultrasphere ODS (5  $\mu\text{m}$ ) Altex (Beckman)  
4.6 mm (i.d.) x 250 mm
- pre-column: Brownlee Labs RP 18 (5  $\mu\text{m}$ )
- eluent A:  $\text{CH}_3\text{CN}$  10% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  90% } pH 6.0
- eluent B:  $\text{CH}_3\text{CN}$  70% } adjusted at  
(2.5 g/l)  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  30% } pH 6.0

elution: linear gradient from 5% to 60% of  
eluent B in eluent A, in 40 min

flow rate: 1.8 ml/min

5

U.V. detector: 254 nm

internal standard: Teicoplanin A<sub>2</sub> component 2  
(Gruppo Lepetit S.p.A.)

10

E) Molecular weight of about 1568 as determined by  
FAB-MS

F) acid functions capable of forming salts

15

G) amino function capable of forming salts

H) no mannose unit linked to the core moiety.

20

Antibiotic A 40926 N-acylaminoglucuronyl aglycon  
factor B<sub>1</sub> (in the non-addition salt form) has the  
following characteristics:

25

has molecular weight of about 1568 as determined by  
FAB-MS and substantially the same physico-chemical  
characteristics reported above for antibiotic  
A 40926 N-acylaminoglucuronyl aglycon factor B<sub>0</sub>  
except that it has a triplet at 0.84  $\delta$  ppm  
attributable to the methyl group of an n-propyl  
function in the NMR system reported above and a  
retention time relative to Teicoplanin A<sub>2</sub> component  
2 of 1.32 in the system reported above.

35

The following "preparations" are an example of the way in which antibiotic A 40926 N-acylamino-glucuronyl aglycon complex and the factors thereof can be prepared:

5  
Preparation 1:

Preparation of antibiotic A 40926 N-acylamino-glucuronyl aglycon complex AB

10

- a) Antibiotic A 40926 complex AB (prepared substantially by following the procedure of Example 3 of EP-A- 177882) (750 mg) is dissolved in 150 ml of a mixture dimethylsulfoxide (DMSO) /37% (w/w) hydrochloric acid (HCl), 9:1 (v/v) and the reaction mixture is heated to about 65°C.

15

The reaction course is monitored by HPLC and when the starting materials are completely reacted (after about 5 h) the reaction is quenched with cold water (600 ml) and the pH of the resulting mixture is adjusted to about 7.5.

20

This mixture contains a mixture of the compounds of the title which is separated into its two major components by affinity chromatography according to the following procedure:

25

- b) The aqueous mixture obtained above (750 ml) is applied to a Sepharose-D-Alanyl-D-Alanine chromatography column prepared as described in EP-A- 177882 and EP-A- 122969, Example 1.A) (100 ml of swollen resin in 10 mM TRIS.HCl pH 7.5 buffer; bed height 10 cm).

30

0.05 M  $\text{NH}_4\text{OH.HCl}$  pH 7.5 containing 2 M NaCl (200 ml) (buffer B) is passed through the column; then A 40926 aglycon is selectively removed from

35

the column by eluting with 0.05 M  $\text{NH}_4\text{OH.HCl}$  pH 9.5 containing 2 M NaCl (1500 ml) (buffer C).

N-Acylaminoglucuronyl aglycon complex AB is then eluted with 0.1 M aqueous ammonia (buffer D).

5 The eluted fractions are then pooled according to their antibiotic content adjusted to about pH 7.5 and each antibiotic containing solution is chromatographed on a Sepharose-D-Alanyl-D-Alanine column (100 ml of swollen resin in 10 mM TRIS.HCl pH 7.5  
10 buffer; bed height 10 cm). Distilled water is passed through the column until the inorganic salts are washed out. The antibiotics are then eluted with 0.1 N aqueous ammonia. These eluted fractions, pooled according to their antibiotic content, are  
15 concentrated to a small volume under reduced pressure by azeotropic distillation with n-butanol and lyophilized yielding respectively 201 mg of N-acylaminoglucuronyl aglycon complex AB and 236 mg of A 40926 aglycon.

20

By repeating the same experiment described above but using a mixture DMSO/37% HCl 95:5 at about 40°C for about 5 days the yield in N-acylaminoglucuronyl aglycon complex AB increases of about 15% while the  
25 yield in A 40926 aglycon is reduced accordingly.

25

By repeating these experiments starting from antibiotic A 40926 complex, antibiotic A 40926 factor A, antibiotic A 40926 factor B, antibiotic  
30 A 40926 factor B<sub>0</sub>, antibiotic A 40926 factor B<sub>1</sub>, antibiotic A 40926 factor PA and antibiotic A 40926 factor PB substantially the same results are obtained (i.e. the yields vary in the range  $\pm 5\%$ ). In particular, starting from antibiotic A 40926  
35 factor A, or factor PA, the product which is

obtained is antibiotic A 40926 N-acylaminoglucuronyl aglycon factor A, starting from antibiotic A 40926 factor PB<sub>0</sub>, or factor B<sub>0</sub> the obtained product is antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B<sub>0</sub>, starting from antibiotic A 40926 factor B or PB the obtained product is antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B, which may in turn be separated into factor B<sub>0</sub> and B<sub>1</sub>, and starting from antibiotic A 40926 factor B<sub>1</sub>, antibiotic A 40926 N-acylaminoglucuronyl aglycon factor B<sub>1</sub> is obtained.

15 Preparation 2:

Separation of antibiotic A 40926

N-acylaminoglucuronyl aglycon factors A, B<sub>0</sub> and B<sub>1</sub>

20 Mg of antibiotic A 40926 N-acylaminoglucuronyl aglycon complex AB is dissolved in 1 ml of 18 mM sodium phosphate buffer pH 6.0 containing 10% of acetonitrile. The solution was injected into a HPLC preparative column (7 mm id x 250 mm) Lichrosorb RP18 silanized silica gel (Merck Co.) having 7 micrometer particle size.

25 The column is eluted at a flow rate of 5 ml/min of phase A and B with a linear gradient from 10% to 55% of phase A in 55 min.

Phase A: 18 mM sodium phosphate/CH<sub>3</sub>CN 30/70 brought to pH 6.0 with NaOH.

30 Phase B: 18 mM sodium phosphate/CH<sub>3</sub>CN 90/10 brought to pH 6.0 with NaOH.

The column eluates UV adsorption at 254 nm is recorded and the elution fractions having omogeneous content are collected, separating three groups of

eluates containing antibiotic A 40926  
N-acylaminoglucuronyl aglycon factors A, B<sub>0</sub> and B<sub>1</sub>  
respectively.

The eluates containing the purified antibiotic  
5 A 40926 N-acylaminoglucuronyl aglycon factors of 11  
subsequent chromatographic runs are pooled and desalted  
as usual by loading them on a column of 5 ml swollen  
sepharose-D-Ala-D-Ala (see above). After removing the  
salts with 10 ml of 1 mM HCl followed by 5 x 10 ml of  
10 distilled water, the antibiotic is eluted with 5 x 10 ml  
of 1% w/v aqueous ammonia. The ammonia eluates are then  
separately collected and freeze-dried yielding 15 mg of  
antibiotic A 40926 N-acylaminoglucuronyl aglycon factor  
A, 51 mg of antibiotic A 40926 N-acylaminoglucuronyl  
15 aglycon factor B<sub>0</sub> and 3 mg of antibiotic A 40926  
N-acylaminoglucuronyl aglycon factor B<sub>1</sub> whose  
physico-chemical data and chemical formula are reported  
above in the description.

20

The following examples further illustrate the  
invention and, as such, should not be construed as  
limiting its scope.

25

## Example 1:

Fermentation of Actinoplanes teichomyceticus

- 5        A sample of a frozen stock culture of Actinoplanes teichomyceticus ATCC 31121 is used to inoculate 100 ml of vegetative medium having the following composition:

	Glucose	10	g
10	Peptone	4	g
	Yeast extract	4	g
	MgSO <sub>4</sub>	0.5	g
	KH <sub>2</sub> PO <sub>4</sub>	2	g
	K <sub>2</sub> HPO <sub>4</sub>	4	g
15	Deionized water	1000	ml

- 100 ml of the inoculated medium is incubated 48 hours in a 500 ml Erlenmeyer flask at 28°C on a rotary shaker. 200 ml of this culture is used to inoculate 4 l  
20 of fermentation medium having the following composition:

	Peptone	4	g
	Yeast extract	1	g
	Soybean meal	10	g
25	Malt extract	4	g
	Glucose	5	g
	NaCl	2.5	g
	CaCO <sub>3</sub>	5	g
	Deionized water	1000	ml

30



The inoculated medium is fermented at about 28°C under 0.5 v/v/min steril air flow at about 900 rpm for about 48 h.

- 5        Actinoplanes teichomyceticus ATCC 53649 can be used instead of Actinoplanes teichomyceticus ATCC 31121.

- 10       Example 2:  
         Fermentation of Actinoplanes missouriensis  
         ATCC 23342

- 15       A lyophilized tube containing Actinoplanes missouriensis strain ATCC 23342 is open and aseptically transferred into a slant of oatmeal agar. After a 12 day incubation at 28°C, the culture is suspended in distilled water and inoculated into 10 Erlenmeyer-flasks  
20       each containing 100 ml of medium having the following composition:

	Yeast extract	2	g
	Soybean meal	8	g
25	Dextrose	20	g
	NaCl	1	g
	CaCO <sub>3</sub>	4	g
	H <sub>2</sub> O	1000	ml

- 30       The inoculated medium is incubated 48 hours at 30°C on a rotary shaker at 200 rpm.

- Actinoplanes missouriensis NRRL 15646, NRRL 15647,  
35       ATCC 31683, ATCC 31682, ATCC 32680 or a mixture thereof

in any proportion, can be used instead of Actinoplanes  
missouriensis ATCC 23342.

5

Example 3:

Fermentation of Actinoplanes NRRL 3884

10

A lyophilized tube containing Actinoplanes strain  
NRRL 3884 is open and aseptically transferred into a  
slant of oatmeal agar. After a 12 day incubation at  
28°C, the culture is suspended in distilled water and  
15 inoculated into 10 Erlenmeyer flasks each containing  
100 ml of medium having the following composition:

	Yeast extract	2	g
	Soybean meal	8	g
20	Dextrose	20	g
	NaCl	1	g
	CaCO <sub>3</sub>	4	g
	H <sub>2</sub> O	1000	ml

25

The inoculated medium is incubated 48 hours at 30°C  
on a rotary shaker at 200 rpm.

## Example 4:

## Preparation of de-acyl antibiotic A 40926

## a) Biotransformation of antibiotic A 40926 complex AB

5

Antibiotic A 40926 complex AB (prepared substantially as described in EP-A- 177882) is aseptically added to the fermenting culture prepared substantially as described in Example 1, 2 or 3, 48 hours after inoculum. The biotransformation process is monitored by HPLC analysis of the broth. Glycopeptide antibiotics are purified on Sepharose-D-Alanyl-D-alanine (see EP-A-122969) and are analyzed according to the following HPLC method:

15

Column: Ultrasphere ODS (5  $\mu$ m) 4.6 mm x 25 cm. .  
Altex (Beckman)

Precolumn: Brownlee labs RP18 (5  $\mu$ m)

20

Phase A: 18 mM sodium phosphate buffer/CH<sub>3</sub>CN  
98/2 (v/v)  
brought to pH 6.0 with NaOH

Phase B: 18 mM sodium phosphate buffer/CH<sub>3</sub>CN  
30/70 (v/v)  
brought to pH 6.0 with NaOH

25

Elution: linear gradient from 5% to  
65% of phase B in 43 min

Flow rate: 1.8 ml/min

Detection: UV 254 nm

30

The retention time of de-acyl antibiotic A 40926 is in the range 8.3 and 9.

35

The harvesting time is set at about 196 hours after the addition of antibiotic A 40926 complex AB to the medium for Actinoplanes NRRL 3884, about 168

hours for Actinoplanes missouriensis ATCC 23342, ATCC 31683, ATCC 31682, ATCC 32680, NRRL 15646 and NRRL 15647 and about 192 hours for Actinoplanes teichomyceticus ATCC 31121 and ATCC 53649. The deacylation efficiency is substantially similar with any of the above cultures.

b) Recovery and purification:

The harvested broth obtained from the pooled Erlenmeyer flasks is brought to pH 9.5 with NaOH and filtered with Hyflo-FloMa filter aid. The filter cake is discharged while the clear filtrate is adjusted to pH 7.5 with HCl. 10 ml of swollen Sepharose-D-Alanyl-D-Alanine (see above) is added and this mixture is stirred overnight at room temperature. The resin is then recovered by filtration and washed sequentially on the filter with 4 x 40 ml of 40 mM TRIS.HCl buffer (pH 6.5) /2-amino-2-hydroxy-methyl-1,3-propanediol/ and 6 x 40 ml of distilled water. Then, a mixture is eluted from the resin with 3 x 40 ml of 1% (w/v) aqueous  $\text{NH}_4\text{OH}$ . This solution is cooled to about 4°C and brought to about pH 3.5 with  $\text{H}_2\text{SO}_4$ . The precipitate is removed by centrifugation, while the supernatant that contains the biotransformed antibiotic A 40926 in a solution (150 ml) is brought to about pH 7.0 with NaOH and loaded on a column (diameter 1 cm) containing 25 ml of Sepharose-D-Alanyl-D-Alanine swollen in distilled water. The column is eluted sequentially with 50 ml of distilled water and 200 ml of ethanol/water 1/9 (v/v). The antibiotic substance of the title is then eluted with 35 ml of 1% (w/v) aqueous  $\text{NH}_4\text{OH}$ . This solution is concentrated under vacuum and then freeze-dried

yielding 41-45 mg of de-acyl antibiotic A 40926.  
The physico-chemical characteristics are reported  
above in the description.

5 By repeating the same procedure starting from  
antibiotic A 40926 factor A or antibiotic A 40926  
factor B or B<sub>0</sub> the same compound is obtained with  
similar yields.

10

Example 5:

Preparation of antibiotic A 40926 aminoglucuronyl  
aglycon

15

If the procedure of example 4 is repeated starting  
from antibiotic A 40926 N-acylaminoglucuronyl aglycon  
complex AB, antibiotic A 40926 N-acylaminoglucuronyl  
20 aglycon factor A, factor B, factor B<sub>0</sub> or B<sub>1</sub> (prepared  
as described above) antibiotic A 40926 aminoglucuronyl  
aglycon is obtained which has the characteristics  
reported above in the description.

25

Example 6:

Preparation of de-acyl antibiotic A 40926 P

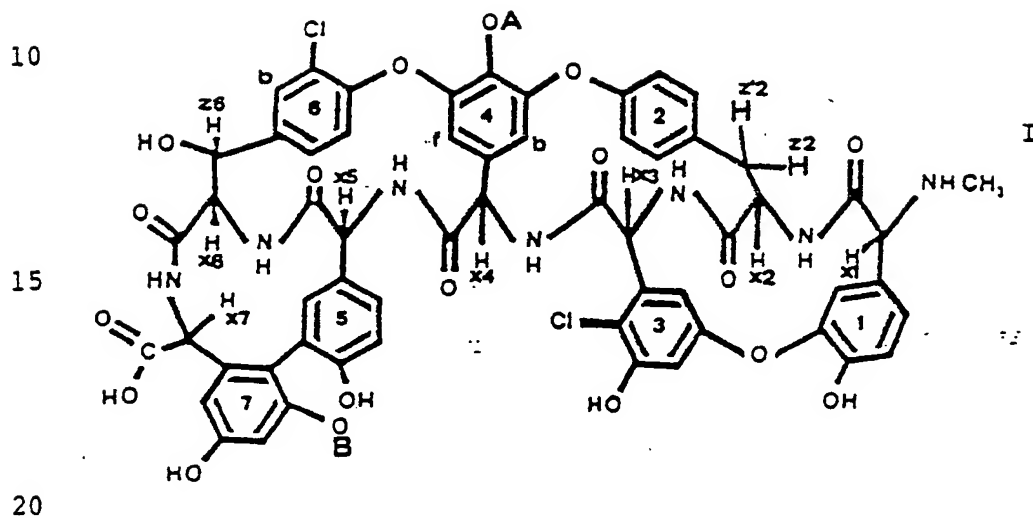
30

By repeating the procedure of example 4 but  
starting from antibiotic A 40926 factor PA or factor PB,  
or a mixture thereof in any proportion and reducing to a  
minimum the permanence of the reaction mass at basic pH

values, de-acyl antibiotic A 40926 P is obtained whose characteristics are as reported above.

## Claims

1. A de-acyl A 40926 antibiotic of formula:



wherein:

- A represents a 2-amino-2-deoxy-beta-D-glucopyranosiduronic acid group and  
 B represents hydrogen, alpha-D-mannopyranosyl or 6-acetyl-alpha-D-mannopyranosyl,

and the addition salts thereof.

2. De-acyl antibiotic A 40926 or an addition salt thereof, which has the following characteristics, in the non addition-salt form:

5

A) ultraviolet absorption spectrum which exhibits the following absorption maxima:

		$\lambda$ max (nm)
10	a) 0.1 M HCl	282
	b) phosphate buffer pH 6.0	281
	c) phosphate buffer pH 7.4	282, 300 (shoulder)
	d) 0.1 M KOH	300

15

B) infrared absorption spectrum which exhibits the following absorption maxima in nujol mull ( $\nu$ ,  $\text{cm}^{-1}$ ):

3700-3100; 3000-2800 (nujol); 1650; 1590; 1505;  
20 1460 (nujol); 1375 (nujol); 1300; 1230, 1210, 1150,  
1060, 1030, 970, 810, 720 (nujol)

C)  $^1\text{H}$ -NMR spectrum which exhibits the following groups of signals (in ppm) at 270 MHz recorded in DMSO  $\text{d}_6$  (hexadeuterodimethylsulfoxide) [7 $\delta$ , ppm; m; (attributions)]

25 2.30, s ( $\text{N-CH}_3$ ); 2.49, s ( $\text{DMSO d}_5$ ); 2.7-3.8, m (sugar CH's); 2.79 m (Z2); 4.08 m (X6); 4.33 s (X1);  
30 4.37 d (X5); 4.37 d (X7); 4.86 m (X2); 5.08 s (4f); 5.08 s (Z6); 5.27 s (anomeric proton of mannose); 5.35 d (anomeric proton of aminoglucuronic acid); 5.61 d (X4); 5.86 s (4b);  
35 6.05, d (X3); 7.73 s (6b); 6.45-8.49 (aromatic protons and peptidic NH's)



D) Retention time ( $R_t$ ) of 0.34 relative to Vancomycin (Eli Lilly)

Column: Silanized silica gel Ultrasphere ODS (5  $\mu$ m)  
4.6 mm x 25 cm Altex (Beckman)

5 Isocratic elution with 18 mM sodium phosphate  
buffer/ $\text{CH}_3\text{CN}$  92/8 (v/v)

Flow rate: 1.8 ml/min

Detection: UV 254 nm

10 Internal standard: Vancomycin (Eli Lilly)  $R_t$  8.4  
min

E) molecular weight of 1548 as determined by FAB-MS  
spectroscopy.

15

3. Antibiotic A 40926 aminoglucuronyl aglycon which  
is a compound of claim 1 wherein A is as defined and B  
represents hydrogen, or an addition salt thereof.

20

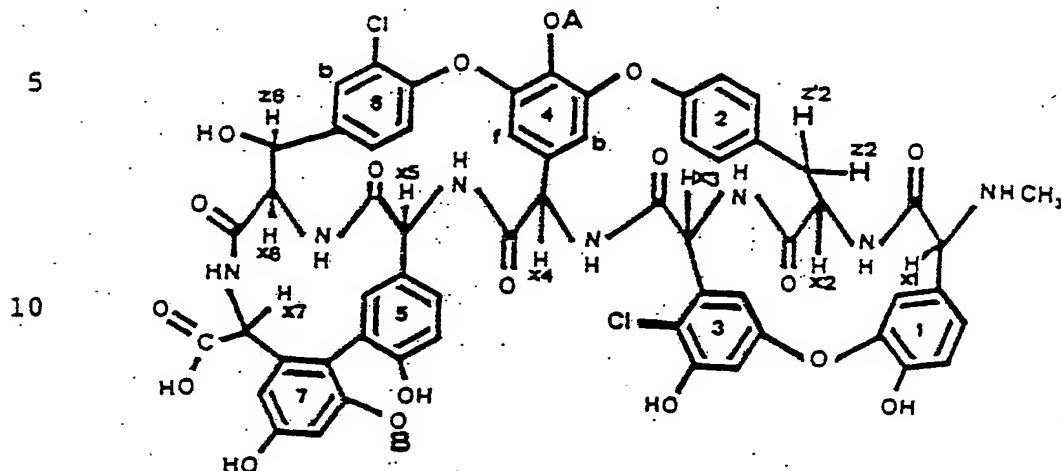
4. De-acyl antibiotic A 40926 P which is a compound  
of claim 1 wherein A is as defined and B represents  
6-acetyl-alpha-D-mannosyl, or an addition salt thereof.

25

5. De-acyl antibiotic A 40926 which is a compound  
of claim 1 wherein A is as defined and B represents  
alpha-D-mannosyl, or an addition salt thereof.

30

6. A process for preparing a compound of claim 1 which comprises treating a compound of formula:



wherein A represents a 2-deoxy-2-(C<sub>11</sub>-C<sub>12</sub>) acylamino-  
-beta-D-glucopyranosiduronic acid group and B represents  
hydrogen, alpha-D-mannosyl or 6-acetyl-alpha-D-mannosyl,  
an addition salt thereof and/or a mixture thereof in any  
proportion, with a growing culture of a strain of the  
genus *Actinoplanes*.

7. A process according to claim 6 wherein the  
strain of the genus *Actinoplanes* is selected from  
*Actinoplanes teichomyceticus* ATCC 31121, *Actinoplanes*  
*teichomyceticus* ATCC 53649, *Actinoplanes*  
*NRRL 3884*, *Actinoplanes missouriensis* ATCC 23342, *Actinoplanes*  
*missouriensis* NRRL 15646, *Actinoplanes missouriensis*  
*NRRL 15647*, *Actinoplanes missouriensis* ATCC 31683,  
*Actinoplanes missouriensis* ATCC 31682, *Actinoplanes*  
*missouriensis* ATCC 32680, and a mutant or variant  
thereof which retain the deacylating capability of the  
parent strain.

8. A process according to claim 6 or 7 wherein a washed mycelium, a cell-free extract or concentrate is used instead of the growing culture of the deacylating microorganism.

5

9. A process according to claim 6, 7 or 8 wherein the reaction temperature is between 20°C and 40°C.

10

10. A process according to claim 8 wherein the reaction temperature is between 25°C and 50°C.

15

11. A process according to claim 6 for preparing de-acyl antibiotic A 40926 from antibiotic A 40926 complex, factor A, B or B<sub>0</sub>.

20

12. A process according to claim 6 for preparing antibiotic A 40926 aminoglucuronyl aglycon from antibiotic A 40926 N-acylaminoglucuronyl aglycon complex, factor A, B or B<sub>0</sub>.

25

13. A compound according to claim 1, 2, 3, 4 or 5 for use as a medicine.

30

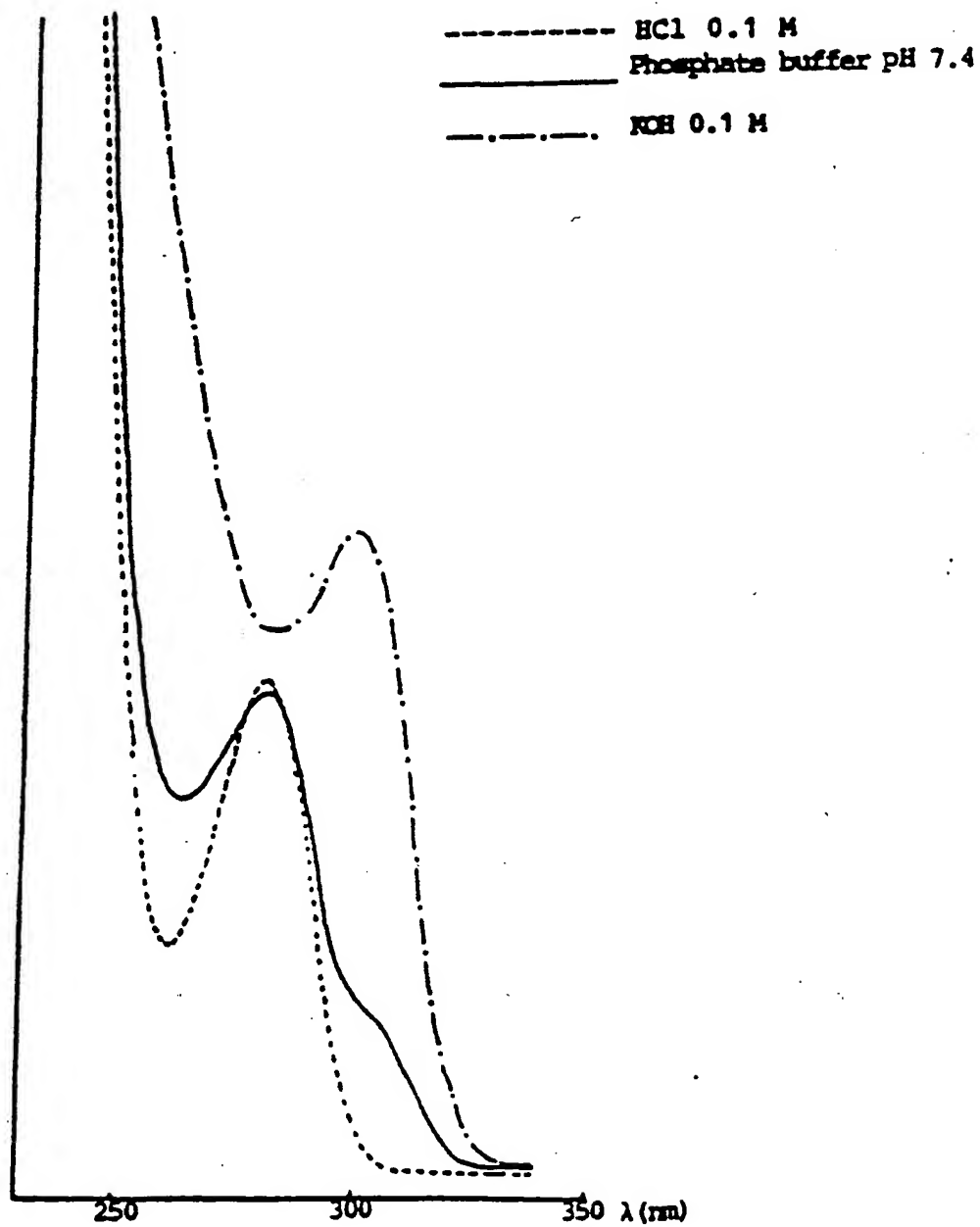
14. Use of a compound of claim 1, 2, 3, 4 or 5 for preparing a medicament for antimicrobial treatment.

35

15. A pharmaceutical composition which contains a compound of claim 1, 2, 3, 4 or 5 in admixture with a pharmaceutically acceptable carrier.

## U.V. SPECTRUM OF DE-ACYL ANTIBIOTIC A 60926

FIG. 1



## I.R. SPECTRUM OF DE-ACYL ANTIBIOTIC A 40926

FIG. 2

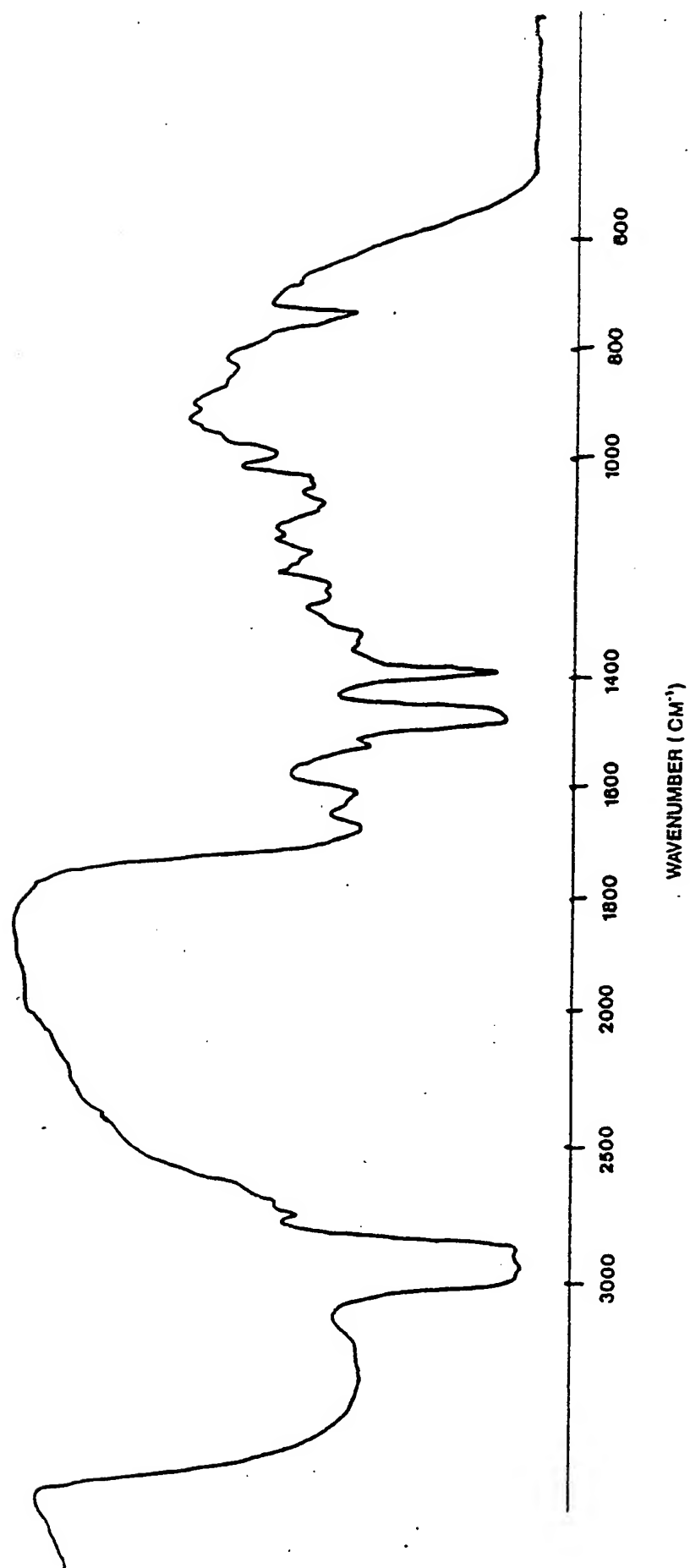
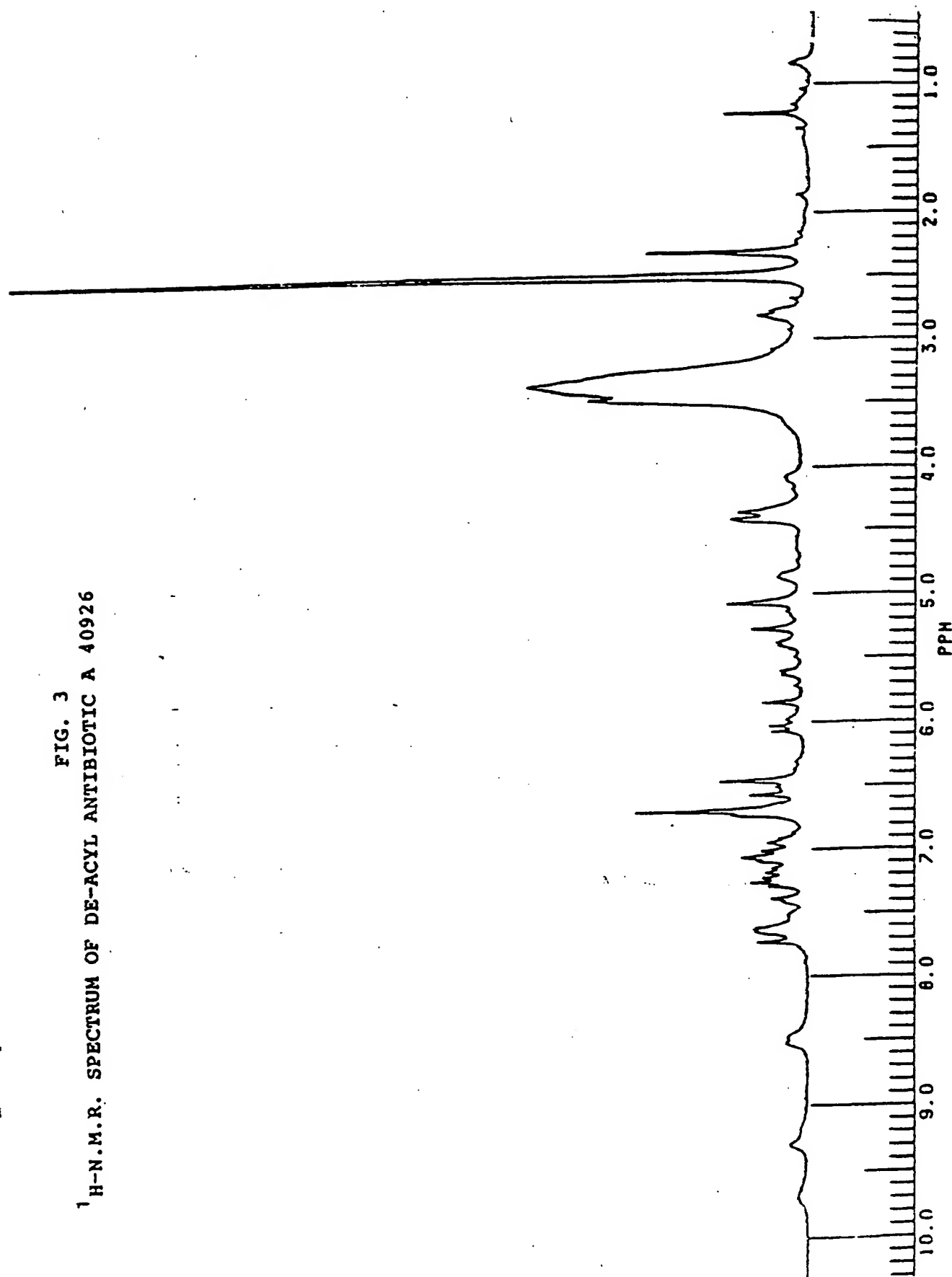


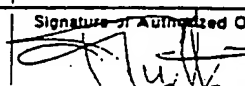
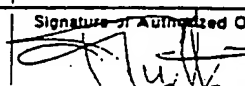
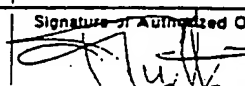
FIG. 3  
<sup>1</sup>H-N.M.R. SPECTRUM OF DE-ACYL ANTIBIOTIC A 40926



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 87/00588

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> :    C 07 K 9/00; A 61 K 37/02											
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC <sup>4</sup></td> <td style="padding: 5px;">C 07 K 9/00; A 61 K 37/00</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 07 K 9/00; A 61 K 37/00					
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IPC <sup>4</sup>	C 07 K 9/00; A 61 K 37/00										
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>10</sup></th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0177882 (LEPETIT) 16 April 1986 see pages 42,43 cited in the application --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,13</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0055071 (ELI LILLY) 30 June 1982 see page 22, lines 12-30; claim 1  -----</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,13</td> </tr> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	A	EP, A, 0177882 (LEPETIT) 16 April 1986 see pages 42,43 cited in the application --	1,13	A	EP, A, 0055071 (ELI LILLY) 30 June 1982 see page 22, lines 12-30; claim 1  -----	1,13
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A	EP, A, 0055071 (ELI LILLY) 30 June 1982 see page 22, lines 12-30; claim 1  -----	1,13									
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>10</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>											
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">13th January 1988</td> <td style="text-align: right; padding: 5px;">19 FEB 1988</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="text-align: center; padding: 5px;">  P.C.G. VAN DER PUTTEN         </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	13th January 1988	19 FEB 1988	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN	
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13th January 1988	19 FEB 1988										
International Searching Authority	Signature of Authorized Officer										
EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN										

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

EP 8700588

SA 18948

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/02/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0177882	16-04-86	JP-A- 61148188	05-07-86
		AU-A- 4819585	17-04-86
EP-A- 0055071	30-06-82	GB-A, B 2089815	30-06-82
		JP-A- 57129693	11-08-82
		AU-A- 7860281	24-06-82
		US-A- 4461723	24-07-84
		CA-A- 1172187	07-08-84
		US-A- 4637981	20-01-87